ISOLATION AND CHARACTERIZATION OF ALLOPHYCOCYANIN II FROM THE THERMOPHILIC BLUE-GREEN ALGA *MASTIGOCLADUS LAMINOSUS* COHN

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1. Introduction

Phycocyanins and phycoerythrins are the primary absorbers of light used in photosynthesis by red and blue-green algae. Besides their function as energy transfer pigments [1], they seem to act as an electron-directing agent in trans-membrane migration of electrons [2,3]. Generally it has been assumed that the energy absorbed by phycoerythrin is passed through C-phycocyanin to chlorophyll. Recently Gantt and Lippschulz reported that allophycocyanin seems to be the pigment through which energy trapped by phycobiliproteins is funneled to the chloroplast lamellae. Up to now the phycoerythrins and C-phycocyanins of a number of red and blue-green algae have been purified and characterized with respect to their gross chemical properties [5–8]. However only very little information about allophycocyanins is available [6,9].

It therefore seemed of interest to us to study this particular accessory pigment and to compare it with C-phycocyanin extracted from the same organism [8]. In this report we show the isolation and characterization of allophycocyanin II from the thermophilic blue-green alga *Mastigocladus laminosus.*

2. Materials and methods

Alcohol dehydrogenase (horse liver), carbonic anhydrase (bovine erythrocytes), lysozyme (hen egg white) and hemoglobin (bovine) were obtained from Serva, Heidelberg, Germany; Carboxypeptidase A and Pepsin from Wortington; β-lactoglobulin from Mann Research, New York, USA, and myoglobin (equine heart) from Pentex, Kaukakee, Illinois, USA. Ampholyte came from LKB, Sweden. All reagents were of analytical grade. The cellogel-electrophoresis strips came from Chemetron, Milan, Italy. *M. laminosus* cells were cultured in the water of a hot spring near Reykjavik, Iceland [10]. All purification steps were performed routinely at 4°C in the dark.

Cells (300 g wet weight) were suspended in 900 ml 0.02 M phosphate buffer, pH 8.0 (this buffer was used in all the following purification steps) and homogenized in a mixer (MSE-Ato Mix) for 5 min. The slurry was passed three times through a Menton-Gaulin homogenizer Model 15 M. The cell debris was centrifuged for 3 hr at 43 000 *g* in a Sorvall centrifuge Model RC-2B. The supernatant was concentrated in a Diaflo ultrafiltration cell (membrane: UM 20E) to a volume of 250 ml. This solution was brought to 30% saturation with ammonium sulfate, left standing for 30 min and centrifuged at 43 000 *g* for 1 hr (the same centrifugation conditions were used for all the ammonium sulfate steps). The resulting supernatant was brought to 50% ammonium sulfate saturation left to stand for 30 min and centrifuged. The pellet was resuspended in 200 ml phosphate buffer and dialyzed for 48 hr against 4 litres of the same buffer. Half of the dialyzed solution was passed through a 10 × 100 cm Sephadex G-150 column equilibrated in the same buffer. The biliprotein containing fractions were pooled, concentrated (Diaflo) to 170 ml brought to 20% saturation with ammonium sulfate and left stand-

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ing for 30 min. After centrifugation, the supernatant was dialyzed for 48 hr against phosphate buffer. Half of the protein solution was transferred to a 5 X 100 cm DEAE-Sephadex A-50 column in equilibrium with phosphate buffer. Elution was performed with a linear gradient of 0.18 – 0.4 M NaCl in 0.02 M phosphate buffer pH 8.0 (total volume 2.2 litres). The collected fractions of allophycocyanin II were concentrated and dialyzed for 24 hr against 2 litres of phosphate buffer. Final purification was achieved by gel filtration with recycling on a 2.5 X 100 cm Sephadex G-100 column.

The purity of the protein was monitored with polyacrylamide disc electrophoresis using the method of Ornstein and Davies [11]. For gels at pH 7.5 in Tris–HCl buffer the Tris–barbiturate buffer system according to Williams ans Reisfeld [12] was used. The same system was also used for urea disc gels (8M) at pH 7.5. The protein was treated with 8M urea, 0.005 M cystein (2½ hr, 37°C).

Ion focussing was carried out according to the LKB 8100 Ampholine Instruction Manual. The column volume was 110 ml, the ampholyte used was in the pH range of pH 3.0–pH 5.0. For subunit separation about 10 mg of pure allophycocyanin II in 0.02 M phosphate buffer pH 8.0, containing 8 M urea and 0.01 M β-mercaptoethanol were incubated for 2½ hr at 37°C and applied to a 2.5 X 45 cm DEAE-Sephadex A-50 column equilibrated in the same buffer. Elution was performed at room temperature with a linear gradient of 0.05 M–0.3 KCL in phosphate buffer (total volume 400 ml). The fractions containing the α and β subunit were pooled separately and dialyzed for 96 hr against 100 times their volume of phosphate buffer. As a second method preparative urea gel electrophoresis was used.

Electrophoresis on cellogel strips of urea-treated allophycocyanin II and of the subunits (9M urea, 2½ hr at 37°C) was carried out at pH 7.2 (0.2 M potassium phosphate, 0.0001 M EDTA, 0.01 M β-mercaptoethanol and 6M urea). The cellogel strips were stained as described by Heil and Zillig [13]. Molecular weight determinations in sodium dodecyl sulfate (SDS) gels were performed as described by Weber and Osborn [14]. Amino acid analyses of acid hydrolysates (6 N HCl, 0.005% phenol, 110°C, 24, 48, 72 hr) were performed on a Beckman amino acid analyzer 121 C [15]. Half cystein was determined as cysteic acid after performic acid oxidation as described by Hirs [16] and tryptophane after hydrolysis in the presence of thioglycolic acid (4%) [17]. All visible and UV spectra were done in 0.02 M phosphate buffer pH 8.0 at room temperature on a Beckman spectrophotometer Acta V. Fluorescence spectra were performed under the same conditions on a Baird Atomic Fluorescence spectrophotometer SF 1-Fluorispec.

3. Results and discussion

30–50% Ammonium sulfate fractionation of the crude extract removed part of the chlorophyll and a large amount of phycoerythrin. On Sephadex G-150 the biliproteins were separated from remaining chlorophyll and carotinoids. The following 20% ammonium sulfate fractionation was effective in removing much of the predominant C-phycocyanin. The different biliproteins allophycocyanin I, phycoerythrin, C-phycocyanin, allophycocyanin II [8] could be separated by ion exchange chromatography on DEAE-Sephadex A-50 (fig. 1). Complete purification of allophycocyanin II (removal of a minor contamination) was achieved by recycling on Sephadex G-100. The yield from 300 g algal cells (wet weight) was generally between 30 and 40 mg of pure allophycocyanin II.

Purified allophycocyanin II showed a single band on polyacrylamide disc electrophoresis in the absence and in the presence of sodium dodecyl sulfate (fig. 2a). After isoelectric focussing the protein eluted as a

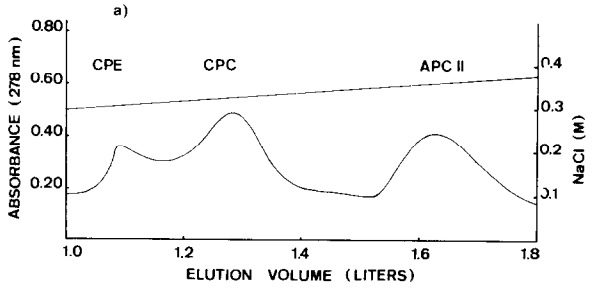

Fig. 1. Purification of allophycocyanin II. a) Chromatography on DEAE-Sephadex A-50 (5 X 100 cm) of the biliprotein fraction after Sephadex G-150 and ammonium sulfate fractionation (0.02 M phosphate buffer pH 8.0, linear NaCl gradient 0.18–0.4 M, total volume 2,1 litres); CPE: C-phycoerythrin; CPC: C-phycocyanin; APC: Allophycocyanin.

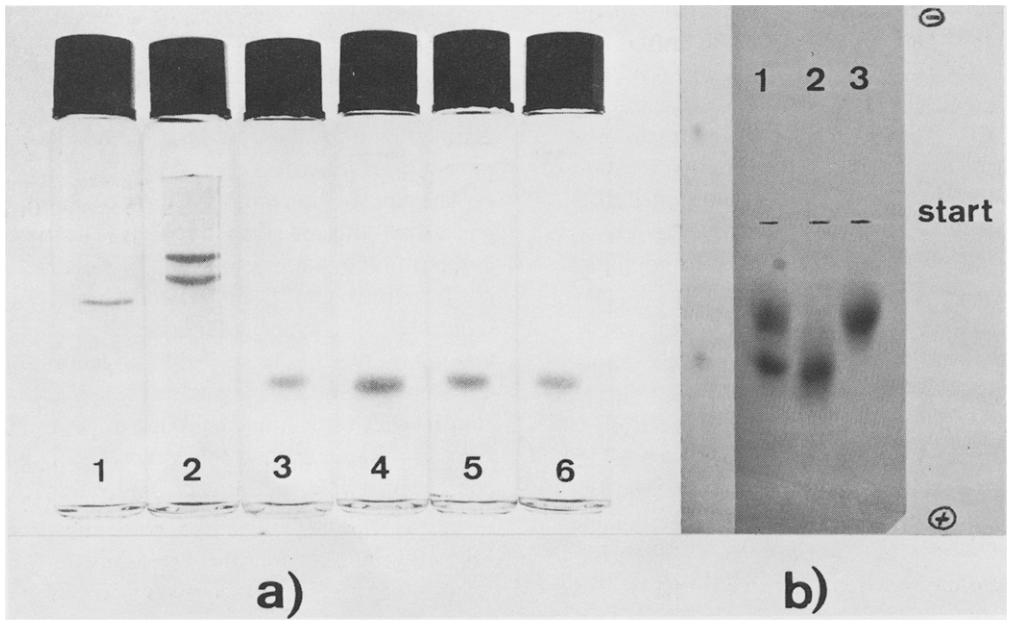

Fig. 2. Electrophoresis of allophycocyanin II and of the subunits. a) Polyacrylamide gel electrophoresis: 1) allophycocyanin II, gel 7,5%, pH 7.5, 2) subunit separation, 8M urea gel 7,5%, pH 7.5, 3) allophycocyanin II, 0.1% SDS, gel 10%, pH 7.0, 4) α-subunit, 0.1% SDS, gel 10%, pH 7.0, 5) β-subunit, 0.1% SDS, gel 10%, pH 7.0, 6) mixture of α- and β-subunit, 0.1% SDS, gel 10%, pH 7.0. b) Cellogel electrophoresis in the presence of 6 M urea: 1) allophycocyanin II, 2) α-subunit, 3) β-subunit.

single band. The isoelectric point was determined to be at pH 4.65 which is higher than the one described by Glazer et al. [9] and lower than the one for C-phycocyanin (4.75 [8]). Upon cellogel electrophoresis in urea as well as urea disc gel electrophoresis allophycocyanin II was separated into two subunits (fig. 2b). This is in agreement with the results of Glazer et al. [9] but not with those of Bennett et al. [6] who only found one subunit. Complete and preparative separation of the α- and β-subunit was achieved by

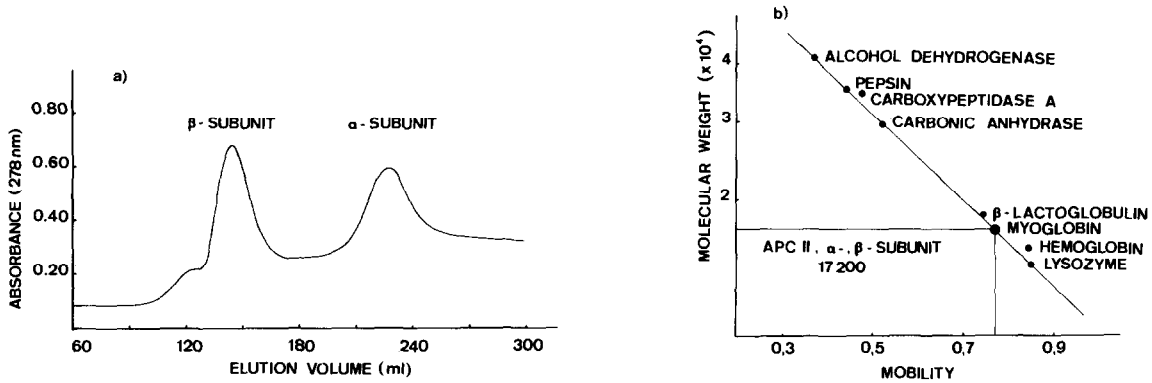

Fig. 3. Subunit separation and molecular weight determination. a) Separation of the α- and β-subunits of allophycocyanin II by chromatography on DEAE Sephadex A-50 (2,5 × 45 cm) in the presence of 8M urea, 0.01 M β-mercaptoethanol (0.02 M phosphate buffer pH 8.0, linear KCl gradient 0.05–0.3, total vol 400 ml). b) Determination of the molecular weight of allophycocyanin II and its subunits by gel electrophoresis in SDS.

ion exchange chromatography in the presence of 8M urea and 0.01 M β-mercaptoethanol (fig. 3a).

The molecular weight of both the α and β subunit, as measured by gel electrophoresis in sodium dodecyl sulfate, is 17 200 (fig. 3b). This is different from the results of Glazer et al., who found values of 15 500 and 17 500. The blue band in each of the unstained SDS gels indicates that both of the allophycocyanin II subunits contain at least one blue bilin chromophore. Assumption of a 1:1 ratio for the two subunits (see amino acid analyses) leads to a minimum mol.wt. of 34 400 for allophycocyanin II.

Determination of the NH_2-terminal residues of allophycocyanin II and its subunits was unsuccessfully tried by the dansyl chloride method for proteins [18] as well as by the method described by Hartley [19]. As well, no free amino acids were found after incubation with an aminopeptidase. NH_2-terminal sequence determination on the α-subunit on a Beckman sequenator (according to Edman and Begg) was not successful. These negative data lead to the assumption that the NH_2-terminus of the α- and β-subunits of allophycocyanin II is blocked.

The amino acid compositions of allophycocyanin II and its subunits are given in table 1. The overall composition of allophycocyanin II is distinctly different from the one of C-phycocyanin extracted from the same alga. Allophycocyanin II contains over 70 amino acid residues more than C-phycocyanin does. Tryptophane and histidine are lacking in allophycocyanin II while both amino acids are present in C-phycocyanin. Remarkable is the comparatively high content of serine-, methionine-, and valine-residues in allophycocyanin II. Like for most of the C-phycocy-

Table 1
Amino acid composition of native allophycocyanin II and the α- and β-subunit

	α-subunit[a] average	integer	β-subunit[a] average	integer	Total α- and β-subunits (α + β)	Native[b] Allophycocyanin II integer	Native [8] C-Phycocyanin integer
Lysine	7.14	7	8.33	8	15	16	10
Histidine[f]	–	–	–	–	–	–	2
Arginine	9.86	10	8.13	8	18	18	15
Aspartic Acid	15.77	16	15.47	15	31	31	30
Threonine[c]	7.26	7	11.91	12	19	19	16
Serine[c]	11.84	12	11.79	12	24	24	14
Glutamic Acid	17.34	17	12.35	12	29	30	25
Proline[g]	5.24	5	4.18	4	9	12	8
Glycine	15.05	15	12.19	12	27	25	18
Alanine	21.68	22	23.71	24	46	46	42
Valine[d]	14.18	14	11.94	12	26	26	14
Methionine	2.79	3	4.06	4	7	7	3
Isoleucine[d]	12.93	13	10.76	11	24	24	18
Leucine	12.87	13	15.35	15	28	28	23
Tyrosine	7.72	8	11.07	11	19	19	13
Phenylalanine	2.99	3	2.04	2	5	5	6
Tryptophane[f]	–	–	–	–	–	–	1
Cysteic acid[e]		1		2	3	3	3
Total		166		164	330	333	261

[a] Amino acid residues calculated per 17 200 mol. wt. after 24, 48 and 72 hr of hydrolysis. [b] Amino acid residues calculated per 34 400 mol. wt. after 24, 48 and 72 hr of hydrolysis. [c] Extrapolated to zero time of hydrolysis. [d] Extrapolated to 120 hr of hydrolysis. [e] Cysteic acid determined after performic acid oxidation according to Hirs [15]. [f] No histidine and tryptophane residues are present in allophycocyanin II. [g] Discrepancies in proline content of total α- and β-subunits and native allophycocyanin II were due to non-linearity of the analyzer. The latest analyses gave without exception values of 10 prolines for native allophycocyanin II.

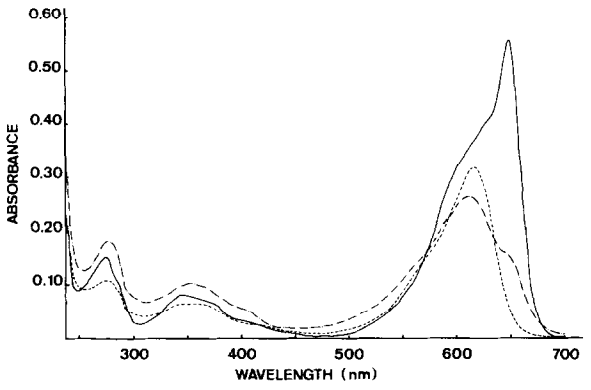

Fig. 4. Absorption spectra of native allophycocyanin II and the α- and β-subunits in 0.02 M phosphate buffer pH 8.0 at room temperature. (——) Allophycocyanin II, (– – – –) α-subunit, (–·–·–·–) β-subunit.

anins a high alanine content and a high ratio of acidic to basic residues holds also true for allophycocyanin II. The most significant difference between the α- and β-subunits lies in the amount of threonine-, glutamic acid-, tyrosine- and cysteic acid residues present.

The absorption maximum of allophycocyanin II is at 650 nm (fig. 4). The shoulders at 595 nm and 625 nm in the absorption spectrum are also described by Bennett et al. [6], but are missing in other published spectra [20,21]. Both, α- and β-subunits have their absorption maximum at 615 nm (C-phycocyanin: α 600 nm; β 618 nm). Allophycocyanin II and the β-subunit show only a very faint fluorescence while the α-subunit has a strong red fluorescence. The fluorescence maximum of allophycocyanin II is at 662 nm. This is in agreement with the value found by O'Heocha [22]. The maxima of the α- and β-subunits are at 630 nm.

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